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PATENT TRADEMARK OFFICE

PATENT
Attorney Docket No. 03806.0517

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	
Francis BLANCHE *et al.*	
Application No.: 09/970,663	Group Art Unit: 1635
Filed: October 5, 2001	Examiner: Brian Whiteman
For: COMPOSITION FOR THE PRESERVATION OF INFECTIOUS RECOMBINANT ADENOVIRUSES	

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

DECLARATION UNDER 37 C.F.R. § 1.131

I, Francis Blanche, state that I am one of the named applicants of the above-identified application and that I am a co-inventor of the subject matter described and claimed therein. Prior to November 16, 1998, we, the co-inventors had completed in France the invention as described and claimed in the above-identified application as evidenced by the following:

1. Exhibit A: Laboratory Notebook Pages 51-55 and 176 (A1-A6) of Francis Blanche, showing, a composition comprising adenoviral particles and a glycerol buffer solution at pH 8.4, wherein the buffer solution does not contain added divalent metal cations or alkali metal cations. See pages 52-53 (A2-A3), formulation #2, for example, comprises Tris/HCl and 10% glycerol at pH 8.4 (hereinafter referred to as "formulation #2".) The addition of adjuvants, such as sucrose or Tween20 is shown, for example, at page 176, formulations C and D. Formulation #2 is shown to be

useful for preserving adenoviruses. See page 55 (A5), stable viral titer after 15 days of storage in formulation #2. Some compositions were tested for stability after –20°C or 4°C storage, indicating that the –20°C frozen viral compositions were thawed to test viability. See page 176 (A6), last three lines from the bottom.

2. The present specification at page 17, first formulation in the Table, shows a formulation identical to formulation #2 of Exhibit A;

3. Example 3 of the present specification, at pages 18-19, shows that a formulation identical to formulation #2 of Exhibit A has a stable viral titer after 15 days of storage, similar to the 15-day storage stability of formulation #2 shown on page 55 (A5) of Exhibit A.

While the dates have been redacted, the undersigned testifies that all experiments described herein were conducted before November 16, 1998.

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that the statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patents issuing thereon.

Dated: March 28, 2003

By: F Blanche
Francis Blanche

08888.0517

EXHIBIT "A" (6 pages)

U.S. Application No. 09/970,665
Attorney Docket # 08888.0517

051

ESSAI N° _____

CEL 02051

ESSAIS FORMULATIONS STABILITE

BUT : Observer la stabilité ou la précipitation éventuelle du virus Y28 dans différentes formulations.

MATERIEL VIRAL ETUDIE :

Solution virale Y28 produite en Cell Cube à l'échelle 8 Mer par l'équipe JF Chaubard et purifiée par chromatographie échangeuse d'anions, conservée dans le Tris 20mM pH8, $MgCl_2$ 1mM, NaCl 500mM et glycerol 10%.
Le virus purifié titre $3,94.10^{11}$ pv/ml.

PREPARATION DES DIFFERENTS TAMPONS ETUDIES :

1. Solutions mères :

	SOLUTIONS MERES :	PREPARATIONS :
A	Tris / HCl pH 8,4 à 500mM	10,07g Tris base + 6,60g Tris/Hcl dans 250ml eau PPI (Tris base ref: T8524 et Tris HCL ref:T7149)
B	Sucrose à 50g/100ml	250g de sucrose dans 500ml d'eau PPI
C	NaCl 5M	Sigma - Aldrich ref.S150
D	$MgCl_2$ 1M	Sigma - Aldrich ref.M1028
E	Glycerol	Sigma - Aldrich ref.G5516
F	D-Mannitol	Sigma - Aldrich ref.M9647
G	Tween 20	Sigma - Aldrich ref.P8074
H	Tampon borate pH 7.4 100mM	Acide borique 100mM + NaOH 0,1N
I	Tampon phosphate pH 7.4 10mM	130mg KH_2PO_4 + 705mg K_2HPO_4 dans 500ml eau PPI

A-1

052

ESSAI N° _____

BI

2. Formulations :

	SOLUTIONS MERES :									
ESSAIS :	A	B	C	D	E	F	G	H	I	Eau PPI
1	20ml									qsp 500ml
2	20ml				+ 50ml					qsp 500ml
3	20ml	50ml		0,5ml						qsp 500ml
4	20ml	50ml								qsp 500ml
5	20ml	50ml		0,5ml		25g				qsp 500ml
6	20ml	50ml	15ml	0,5ml		25g				qsp 500ml
7	20ml	50ml					0,5ml			qsp 500ml
8	20ml	50ml		0,5ml			0,5ml			qsp 500ml
9		50ml		0,5ml				50ml		qsp 500ml
10					+ 50ml				500ml	
11	Solutionvirale obtenue au 2ème rinçage lors de la diafiltration finale. Titre = $2.88.10^{11}$ pv/ml dans DPBS/ NaCl 150mM /glycerol 10%									

3 Résumé des formulations étudiées :

Voir tableau ci-après.

A-2

U.S. Application No. 09/970,663

053

Y28 CELL CUBE 8MER

ESSAIS DE FORMULATIONS

ESSAI N° ____

ESSAIS	Tris 20mM pH 8,4	NaCl 150mM	MgCl2 1mM	Sucrose 5%	Tween20 0,1%	Mannitol 5%	Glycerol 10%	DPBS	Tampon 10mM borate pH7,4	Tampon 10mM pH7,4 phosphate
1	+	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	+	[redacted]		+
2	+	[redacted]	+	+	[redacted]	[redacted]	[redacted]	[redacted]		+
3	+	[redacted]	[redacted]	+	[redacted]	[redacted]	[redacted]	[redacted]		
4	+	[redacted]	+	+	[redacted]	+	[redacted]	[redacted]		
5	+	[redacted]	+	+	[redacted]	+	[redacted]	[redacted]		
6	+	+	[redacted]	+	+	[redacted]	[redacted]	[redacted]		
7	+	[redacted]	+	+	+	[redacted]	[redacted]	[redacted]	+	
8	+	[redacted]	+	+	[redacted]	[redacted]	[redacted]	[redacted]		+
9	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	+	[redacted]		
10	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	+	+		
11	[redacted]	+	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]		

A-3

PAGE 3 of 6

054

ESAI N° _____

St

MATERIELS UTILISES :

→10 PD 10 pour la diafiltration équilibrées avec 5 x 5ml de tampon étudié.

→Ultrafree 15 ml avec membrane Biomax 100 Kd (Millipore) (2x pour chaque essai).

→Centrifugeuse réglée à 1500 tr/mn.

MISE EN OEUVRE :

OPERATIONS :	POUR CHAQUE ESSAI :
DIAFILTRATION :	10 PD10 x 2,5ml de solution virale Y28 à $3,94.10^{11}$ pv/ml. Elution par 10PD10 x 3,5ml du tampon étudié.
CONCENTRATION :	2 Ultrafree 15ml 100Kd remplie à 15ml puis rechargés avec 2,5ml de solution virale diafiltrée. Soit 17,5ml concentrés à 500µl (x2). (soit une concentration à ≈ 1.10^{12} pv/ml.)
RECUPERATION ET FILTRATION 0,2µm :	Récupération en pool des 2 Ultrafree pour chaque essai. Filtration sur filtres Millex 0,2µ non stériles. Stockage dans tubes en verre stériles.
ALIQUOTAGE : (t=0)	→ 100µl dans tube Eppendorff congelé à -26°C par essai. → 20µl + 980µl tampon chp anal. pour dosage. → env.900µl conservés à +4°C pour étude de stabilité. → env.100µl de la solution virale Y28 sortie chromato initiale est congelé à -26°C.
TEMOINS PBS/glycerol 10% :	directement concentré à 1.10^{12} pv/ml. récupéré en aliquoté comme les autres essais.

A-4

ESSAI N° _____

DOSAGES CLHP ANALYTIQUE:

ESSAIS/TAMPONS	TITRE pV/ml J=0	APPARENCE DE L'ECHANTILLON	TITRE pV/ml J=15	OBSERVATIONS CLHP du dosage J=15	TITRE pV/ml J=20 (*) Observations CLHP Apparence échantillon	TITRE pV/ml J=22 (*) Observations CLHP Apparence échantillon
Tampon 1 Tris 20mM	$4,97.10^{12}$	normale à j=15	non filtré : $1,0.10^{12}$ filtré 0,2µm: $9,1.10^{11}$	le retour pic adéno traîne nbre plateaux:12000 asymétries:1,25 et 1,5	non dosé normale	—
Tampon 2 Tris+glycérol	$7,71.10^{12}$	normale à j=15	non filtré : $8,12.10^{12}$ filtré 0,2µm: $7,96.10^{12}$	pic symétrique	non filtré : $7,88.10^{12}$ pic symétrique normale	non filtré : $9,27.10^{12}$ pic symétrique normale
Tampon 3 Tris+$MgCl_2$+sucrose	$6,29.10^{12}$	opacification à j=12 mais non précipité à j=15	non filtré : $2,33.10^{11}$ filtré 0,2µm: $2,09.10^{11}$	montée du pic asymétrique nbre plateaux:32000 asymétries:0,93 et 0,86	non dosé trouble mais non ↓	—
Tampons 4 Tris+sucrose	$6,31.10^{12}$	normale à j=15	non filtré : $5,83.10^{12}$ filtré 0,2µm: $5,7.10^{12}$	pic symétrique	non filtré :$1,87.10^{12}$ nbre plateaux:14000 asymétries:1,28 et 1,42 normale	non filtré :$1,09.10^{12}$ nbre plateaux= 4000 asymétries:0,87 et 0,68 normale
Tampon 5 Tris+$MgCl_2$+sucrose+mannitol	$5,84.10^{12}$	normale à j=15	non filtré : $1,85.10^{12}$ filtré 0,2µm: $1,47.10^{12}$	le retour pic adéno traîne nbre plateaux:17000 asymétries:1,08 et 1,12	non dosé normale	—
Tampon 6 Tris+NaCl+$MgCl_2$+sucrose+mannitol	$6,48.10^{12}$	précipité à j=7	non filtré : non dosé filtré 0,2µm:	—	—	—
Tampon 7 Tris+sucrose+tween	$6,22.10^{12}$	normale à j=15	non filtré : $9,53.10^{11}$ filtré 0,2µm: $9,31.10^{11}$	sommet du pic arrondi nbre plateaux:9000 asymétries:0,95 et 0,83	non dosé normale	—
Tampon 8 Tris+$MgCl_2$+sucrose+tween	$7,17.10^{12}$	opacification à j=7 précipité le lendemain	non filtré : non dosé filtré 0,2µm:	—	—	—
Tampon 9 Borate+$MgCl_2$+sucrose	non détecté	virus retenu sur le filtre solution trouble dès le changement de tampon	non filtré : non dosé filtré 0,2µm:	—	—	—
Tampon 10 Phosphate +glycérol	$7,20.10^{12}$	opacification à j=2 précipité le lendemain	non filtré : non dosé filtré 0,2µm:	—	—	—
Tampon 11 DPBS+NaCl+glycérol	$5,37.10^{12}$	précipité à t<1 jour	non filtré : non dosé filtré 0,2µm:	—	—	—

note: pour le pic adéno étalon →nbre plateaux: 32000 /asymétries: 1,1 et 1,16
(*) calcul des titres avec le nouvel étalon.141
Essais Tp2 et Tp4 retirés à J=22 pour test bioactivité par M.Janicot

A.5
Page 5 of 9

176

SSAI N° _____

SUJET : ADENOVIRUS

SUJET

MISE EN PLACE DES ESSAIS DE STABILITE ADENOVIRUS DANS DIFFERENTES FORMULATIONS

Echantillon de départ: 400ml fraction F3 (+10% glycérol) du DEMOBATCH 3 (CC16M-Ad5/CMV/P53/293) ,dosée à $3,6.10^{11}$ pv/ml soit $1,44.10^{14}$ pv pour 400ml.

Tampons étudiés (filtrés 0,22µm):

-Tampon A :Tris 20mM-pH8,4+10% glycérol
-Tampon B :Tris 20mM-pH8,4+5% sucrose
-Tampon C :Tris 20mM-pH8,4+10% glycérol+5% sucrose
-Tampon D :Tris 20mM-pH8,4+5% glycérol+10% sucrose
-Tampon E :Tris 20mM-pH8,4+10% glycérol+1mM $MgCl_2$
-Tampon F :Tris 20mM-pH8,4+ 10% glycérol+150mM NaCl+1mM $MgCl_2$
-Tampon G :Tris 20mM-pH8,4+5% glycérol
-Tampon H :Tris 20mM-pH8,4+10% sucrose

-Tampon I :Acétate d'ammonium 20mM-pH8+10% glycérol
-Tampon J :Acétate d'ammonium 20mM-pH8+5% sucrose

Mise en place des essais :dans labo L3 de recherches/Bt Monod

-1ère étape : concentration de l'échantillon en utilisant 16 Ultrafree 15ml/30Kd membrane biomax (UFV2BTK40 Millipore) ,centrifugation à 1500tr/mn .Premier passage .on amène le volume à 5ml , (il faut environ 30mn pour le passage de 5 ml)
on recharge une deuxième fois les Ultrafree avec 10ml (on tourne à 1760tr/mn-500G)
et on amène le volume total final à 105ml.
on conserve 5ml pour électrophorèse 2D et on effectue un dosage HPLC (d1/10)
on trouve $1,21.10^{12}$pv/ml soit $1,27.10^{14}$pv pour 105ml.

-2ème étape : changement de tampon sur PD10 Pharmacia (4 PD10 par tampon, soit 4 fois 2,5ml du concentrat ou $1,21.10^{13}$pv/tampon) , on récupère 14ml.

-3ème étape : on concentre les éluats PD10 sur un Ultrafree 15ml/30Kd (même réf. que étape 1) ,on amène le volume à <1ml.
on récupère le concentrat et on volume à 1ml avec le filtrat

-4ème étape : on fait subir à chaque échantillon une filtration stérilisante sur un filtre Millipore (Sterile Millex-GV 0,22µm) membrane PVDF .récupération dans un tube stérile.

-5ème étape : sur chaque échantillon de 1ml après filtration →dosage HPLC (d1/50)
pour les échantillons TpA à E ,aliquoter 14 tubes de 50µl .dans tubes stériles,
pour les échantillons TpF à J ,il y a 15 aliquotes de 50µl.
les titres se situent entre $9,8.10^{12}$ et $1,08.10^{13}$ pv/ml (voir cahier DOS-01 page 42)

-6ème étape : les aliquotes de 50µl sont mis ce jour en stabilité à -20°C.
les reliquats soit ~250 à 300µl sont conservés à 4°C .

Il est prévu un dosage pfu (labo D.Faucher) de chaque échantillon →1 tube de 50µl à -20°C

Note : [illegible] les échantillons à 4°C [illegible]

A-6

ENGLISH-LANGUAGE TRANSLATION OF EXHIBIT "A" (6 pages)

TRIAL NO. _____

CEL 02051

FORMULATION TRIALS: STABILITY.

OBJECTIVE: Observe the stability, or possible precipitation, of the Y28 virus in different formulations.

VIRAL MATERIAL STUDIED:

Y28 solution produced in a cell cube on an 8 mer scale by the J.F. Chaubard team, purified by ion exchange chromatography, and preserved in 20mM pH8 TRIS, 1mM $MgCl_2$, 500mM NaCl, and 10% glycerol. The purified virus titrates $3.94.10^{11}$ pv/ml.

PREPARATION OF THE DIFFERENT BUFFER SOLUTIONS USED:

1. Stock solutions:

	STOCK SOLUTIONS:	PREPARATIONS:
A	Tris / HCl pH 8.4 at 500mM	10.07g Tris base + 6.60g Tris/Hcl in 250ml water for injection (Tris base ref: T8524 and Tris HCL ref:T7149)
B	Sucrose at 50g/100ml	250g sucrose in 500ml of water for injection.
C	NaCl 5M	Sigma - Aldrich ref. S150
D	$MgCl_2$ 1M	Sigma - Aldrich ref. M1028
E	Glycerol	Sigma - Aldrich ref. G5516
F	D-Mannitol	Sigma - Aldrich ref. M9647
G	Tween 20	Sigma - Aldrich ref. P8074
H	100mM borate buffer solution pH 7.4	100mM boric acid + NaOH $0_2$1N
I	10mM phosphate buffer solution pH 7.4	130mg KH_2PO_4 + 705mg K_2HPO_4 in 500ml water for injection.

TRIAL NO. _____

2. Formulations:

STOCK SOLUTIONS :										
	A	B	C	D	E	F	G	H	I	Water for injection
TRIAL:										
1	20ml									QS 500ml
2	20ml				+ 50ml					QS 500ml
3	20ml	50ml		0,5ml						QS 500ml
4	20ml	50ml								QS 500ml
5	20ml	50ml		0.5ml		25g				QS 500ml
6	20ml	50ml	15ml	0.5ml		25g				QS 500ml
7	20ml	50ml					0.5ml			QS 500ml
8	20ml	50ml		0.5ml			0.5ml			QS 500ml
9		50ml		0.5ml				50ml		QS 500ml
10					+ 50ml				500ml	
11	Viral solution obtained in the second rinsing during the final diafiltration. Titer = 2.8810^{11} pv/ml in DPBS/1500mM NaCl/glycerol 10%.									

3. Summary of the formulations studied:

See the following tables.

TRIAL NO. _____

Y28 CELL CUBE 8MER

FORMULATION TRIALS

TRIAL	Tris 20mM pH 8(?)	NaCl 150mM	$MgCl_2$ 1mM	Sucrose 5%	Tween20 0.1%	Mannitol 5%	Glycerol 10%	DPBS	10 mM borate pH7.4 buffer solution	10mM pH7.4 phosphate buffer solution
1	+									
2	+						+			
3	+		+	+						
4	+			+						
5	+		+	+		+				
6	+	+	+	+		+				
7	+			+	+					
8	+		+	+	+					
9			+	+					+	
10							+			+
11		+					+	+		

TRIAL NO. _____

MATERIALS USED:

→ 10 PD 10 for diafiltration balanced with 5 x 5ml of the buffer solution studied.

→ 15ml Ultrafree with 100 Kd Biomax (Millipore) membrane (2x for each trial).

→ Centrifuge set at 1500 rev/min.

IMPLEMENTATION:

OPERATIONS:	FOR EACH TRIAL:
DIAFILTRATION:	10 PD10 x 2.5ml of Y28 viral solution at $3.94.10^{11}$ pv/ml. Elution by 10PD10 x 3.5ml of the buffer solution studied.
CONCENTRATION:	15ml 100Kd 2 Ultrafree filled to 15ml and then refilled with 2.5 diafiltrated viral solution. 17.5ml concentrated at 500µl (x2). (or a concentration at ≈ 1.10^{13} pv/ml.)
RECOVERY AND FILTRATION 0.2µm:	Recovery and pooling of the 2 Ultrafree for each trial. Filtration using unsterilized 0.2µ Millex filters. Storage in sterilized glass tubes.
ALIQUOTING : (t=0)	→ 100µl in Ependorff tube frozen at -26°C. → * → 20µl + 980µl anal. HPCL buffer solution for dosing. → About 900µl stored at +4°C to study stability. → About 100µl of the initial chromate emerging Y28 viral is frozen at -26°C.
10% glycerol/PBS Samples:	Frozen directly at 1.10^{13} pv/ml, recovered and aliquoted in the same way as the other trials.

TRIAL NO. _____

ANALYTICAL HPLC MEASUREMENTS:

TRIALS/BUFFERS	TITER pV/ml J=0	SAMPLE APPEARS	TITER pV/ml day=15	OBSERVATIONS HPLC of the dosage, day=15	TITER pV/ml day=20(*) HPLC Observations Sample appears	TITER pV/ml day=22(*) HPLC Observations Sample appears
Buffer 1 Tris 20mM	$4.97.10^{12}$	normal at day=15	unfiltered: $1.0.10^{12}$ filtered 0.2µm: $9.1.10^{11}$	The adeno return peak trails plate number: 12,000 asymmetries: 1.25 and 1.50	undosed normal	—
Buffer 2 Tris*glycerol	$7.71.\ 10^{12}$	normal at day=15	unfiltered: $8.12.10^{12}$ filtered 0.2µm: $7.96.10^{12}$	symmetrical peak	unfiltered: $7.88.10^{13}$ normal symmetrical peak	unfiltered: $9.27.10^{12}$ normal symmetrical peak
Buffer 3 Tris+$MgCl_3$+ sucrose	$6.29.\ 10^{12}$	opacification at day=12[3] but not precipitated at day=15	unfiltered: $2.33.10^{11}$ filtered 0.2µm: $2.09.10^{11}$	asymmetrical rise of the peak plate number: 32,000 asymmetries: 0.93 at 0.86	undosed clouding but not ↓	—
Buffer 4 Tris+sucrose	$6.31.\ 10^{12}$	normal at day=15	unfiltered: $5.83.10^{12}$ filtered 0.2µm: $5.7.10^{12}$	symmetrical peak	unfiltered: $1.87.10^{12}$ plate number: 14,000 asymmetries: 1.28 and 1.42 (normal)	unfiltered: $1.09.10^{12}$ plate number: 4,000 asymmetries: 0.87 and 0.68 (normal)
Buffer 5 Tris+$MgCl_2$+sucrose+mannitol	$5.84.\ 10^{12}$	normal at day=15	unfiltered: $1.85.10^{12}$ filtered 0.2µm: $1.47.10^{12}$	The adeno return peak trails plate number: 17,000 asymmetries: 1.08 and 1.12	undosed normal	—
Buffer 6 Tris+NaCl+$MgCl_2$+sucrose+ mannitol	$6.48.\ 10^{12}$	precipitated at day=7	unfiltered: undosed filtered 0.2µm:	—	—	—
Buffer 7 Tris+sucrose+Tween	$6.22.\ 10^{12}$	normal at day=15	unfiltered: $9.53.10^{11}$ filtered 0.2µm: $9.31.10^{11}$	rounded peak top plate number: 9,000 asymmetries: 0.95 and 0.83	undosed normal	—
Buffer 8 Tris+$MgCl_2$+sucrose+Tween	$7.17.\ 10^{12}$	opacification at day=7 precipitated the next day.	unfiltered: undosed filtered 0.2µm:	—	—	—
Buffer 9 Borate+$MgCl_2$+sucrose	Undetected	virus held on the filter solution clouds once the buffer solution is changed.	unfiltered: undosed filtered 0.2µm:	—	—	—
Buffer 10 Phosphate + glycerol	$7.20.\ 10^{12}$	opacification at day=2 precipitated the next day	unfiltered: undosed filtered 0.2µm:	—	—	—
Buffer 11 DPBS+NaCl+glycerol	$5.37.\ 10^{12}$	precipitated at < 1 day	unfiltered: undosed filtered 0.2µm:	—	—	—

Note: for the adeno return peak measurement standard → plate number 32,000/asymmetries: 1.1 and 1.16.
(*) computation of titers with the new measurement standard: 141

TRIAL NO. _____

SUBJECT: ADENOVIRUS

CONDUCTING ADENOVIRUS STABILITY TRIALS IN DIFFERENT FORMULATIONS

Starting sample: 400ml fraction F3 (+10% glycerol) of DEMOBATCH 3 (CC16M-Ad5/CMV/P53/293) dosed at $3.6.10^{11}$ pv/ml or $1.44.10^{14}$pv per 400ml.

Buffer solutions studied (0.22µm filtered):

-Buffer solution A: Tris 20mM-pH8.4+10% glycerol
-Buffer solution B: Tris 20mM-pH8.4+5% sucrose
-Buffer solution C: Tris 20mM-pH8.4+10% glycerol+5% sucrose
-Buffer solution D: Tris 20mM-pH8.4+5% glycerol+10% sucrose
-Buffer solution E: Tris 20mM-pH8.4+10% glycerol+1mM $MgCl_2$
-Buffer solution F: Tris 20mM-pH8.4+10% glycerol+150mM NaCl+1mM $MgCl_2$
-Buffer solution G: Tris 20mM-pH8.4+5% glycerol
-Buffer solution H: Tris 20mM-pH8.4+10% sucrose

-Buffer solution 1: ammonium acetate 20mM-pH8+10% glycerol
-Buffer solution 1: ammonium acetate 20mM-pH8+5% sucrose

Carrying Out the Trials: At Research Lab L3/Bt Monod

1st Step: Concentrating the sample by using 15ml/30Kd 16 Ultrafee biomax membrane (UFV2BTK40 Millipore), centrifuged at 1500rev/min. First run, volume brought to 5ml (5ml run requires @30 mins).The Ultrafree is filled a second time with 10ml (turning occurs at 1760 rv/min.-500G). The final total volume is brought to 105ml. 5ml is stored for 2D electrophoresis and HPLC (dl/10) measurement occurs. One then finds $1.21.10^{12}$pv/ml, or $1.27.10^{14}$ pv per 105ml.

2nd Step: Changing over the sample to PD10 Pharmacia (4 PD10 by buffer solution, i.e., 4 x 2.5ml of the concentrate or $1.21.10_{13}$pv/buffer solution), 14ml are recovered.

3rd Step: The PD10 eluates are concentrated on a 15ml/30Kd Ultrafree (same ref. as Step 1) and the volume is brought to <1ml. The concentrate is recovered and the volume is increased to 1ml with filtrate.

4th Step: Each sample undergoes a sterilizing filtration on a Millipore film (Sterile Millex-GV 0.22µm) membrane (PVDF). Collected in a sterile tube.

5th Step: On each 1ml sample after filtration →HPLC (d1/50). For samples TpA to E, aliquot 14 tubes of 50µl in sterile tubes. For samples TpF to J, there are 15 aliquots of 50µl. The titers are located between $9.8.10^{12}$ and $1.08.10^{13}$ pv/ml (see Manual DOS-01 page 42).

6th Step: The 50µm aliquots are used while stable at -20ºC. The carry-over, i.e., 250 to 300µl, is stored at 4ºC.

A PFU (D. Faucher Lab) measurement of each sample is provided →1 tube of 50µl at -20˚C.